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Research Article

NEW INSIGHTS ON NEUROPATHOLOGICAL LESIONS PROGRESSION WITH SPECIAL EMPHASIS ON RESIDENCE OF VELOGENIC NEWCASTLE DISEASE VIRAL ANTIGEN IN THE NERVOUS SYSTEM OF EXPERIMENTALLY INFECTED BROILER CHICKENS

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ABSTRACT: New castle disease virus affecting poultry industry resulting in extensive chicken mortalities and economic losses. Evaluation of distribution and severity of neuropathological lesions progressed in different areas of central nervous system was performed in chickens experimentally infected by velogenic Newcastle virus (vNDV). Chickens were inoculated by genotype VII strain (NDV-B7-RLQP-CH-EG-12) via intraocular route at different ages (10, 20 and 30 days old). Serum samples for antibody titer estimation and tissue sections from nervous system were collected for histopathological examination at 1, 3, 5 and 7-days post inoculation (dpi) from all groups. Results indicated that there was variation in antibody titers among different age groups. Encephalitis, myelitis with marked demyelination and axonal spheroids formation were the main neuropathological alterations. Lesions were detected in different areas of central nervous system which vary in distribution and severity among age and duration of infection with complete tract degeneration which was responsible for the developed nervous signs. The experimental infection of broilers by vNDV via intraocular inoculation induced virus dissemination into the central nervous system with progression of neuropathological lesions that were varied in severity, distribution and the onset according to the age of birds with subsequent development of nervous clinical signs and mortalities, in addition the distribution of virus along the CNS clarify the possible pathways of virus dissemination and progression in the neuroparenchyma denoting the neuropathogenesis of the vNDV. The age of bird at the time of infection is a crucial factor in determining the viral replication in neuroparenchyma with subsequently developed neuropathology.

Key words: Newcastle disease, Velogenic, Haemagglutition, Demyelination, Neuropathology, Broilers.

INTRODUCTION

Newcastle disease virus (NDV) has belonged to family Paramyxoviridae, it is an enveloped virus containing linear, non-segmented, negative-sense, single-stranded RNA (Mayo 2002). ND is listed as a disease by the World Organization for Animal Health (OIE) because it can induce high morbidity and mortality in affected birds (Aldous and Alexander 2001).

NDV is classified according to their pathogenicity into velogenic, mesogenic and lentogenic strains, while depend on the tissue tropism and the developed clinical signs, the NDV is classified into viscerotropic velogenic, neurotropic velogenic, mesogenic and lentogenic pathotypes (Aldous and Alexander 2001). High mortalities with nervous and intestinal pathology are recorded with viscerotropic velogenic, neurotropic phenotypes (Alexander *et al.* 2012, Nakamura *et al.* 2008). NDV is considered endemic in Egypt (Mohamed *et al.* 2011, Nabila *et al.* 2014). The pathological lesions in NDV infected chickens varied according to virus phenotype, route, dose and strain of the NDV in addition factors related to host species, age, immune status, co-infection with other organisms and environmental stress (Awan *et al.* 1994).The histopathology of nervous tissue of chickens infected by ND includes non- suppurative encephalitis, neuronal degeneration, perivascular cuffing

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of lymphocytes and glial reaction with vasculitis and demyelination in cerebrum (Bhaiyat *et al*. 1994). In addition, Ecco *et al.* (2011) recorded variation in the histopathological changes developed in brain and spinal cord in chickens experimentally infected with different isolates of NDV. The nervous replication of mesogenic and lentogenic phenotypes of NDV was recorded but the lentogenic strain not recorded and the rate of virus replication differentiate the velogenic strain from the mesogenic one (Moura *et al*. 2016). However, the indices of viral pathogenicity with subsequent related classification of virus virulence did not correlate what occurred typically in animal experiments especially with infection of adult birds by the virus through the natural route of inoculation (Cattoli *et al.* 2011). Thus, further studies must be done to evaluate the effect of bird age on the picture and progression of the disease. The present study investigated the distribution of neuropathological lesions in different areas of cerebrum, cerebellum, spinal cord segments and sciatic nerves in experimentally NDV infected chickens in relation to age and duration of infection as well as clarify the correlation between the developed neuropathology and the NDV antigen residence in different areas of CNS by immunohistochemistry to establish a discussion of the pathogenesis of NDV induced neuropathology and provide a complete neuropathological picture on NDV in different ages of broilers.

MATERIALS AND METHODS

Virus

A local strain of velogenic NDV, genotype VII of NDV(NDV-B7-RLQP-CH-EG-12) was used in this experiment, which was isolated from Kafr El-sheikh governorate, Egypt and was kindly provided by National Laboratory for Veterinary Control on Poultry Production, Animal Health Research Institute. Phylogenetic analysis was performed by partial sequencing of the F gene and it revealed that the isolate belonged to velogenic genotype VII. Sequence was registered at the GenBank under accession number KM 288609.

Infectivity titration in ECE

The propagated NDV viruses' suspensions were titrated in [9-10] day old ECE. Ten fold serial dilutions of the virus in saline contain antibiotic were prepared. Five eggs were inoculated with virus suspension for each dilution via allantoic sac, (0.1 ml per egg). The inoculated embryos were incubated at 37°C and candled twice daily for 6 days. Deaths in the first day were considered as nonspecific deaths. Slide haemagglutination (HA) was applied on the allantoic fluid of inoculated chicken embryos to detect the HA-positive eggs. It was carried out according to the standard method described by Council (1971) for quick detection of haemagglutination in embryonic fluid, 10% washed chicken red blood cell suspension in saline was used. The obtained allantoic fluid was used to determine the egg infectious dose 50 (EID_{50}) according to Reed and Muench (1938) as 10^6 EID_{50} to be used for challenging the chickens.

Experimental design

A total number of 100 one day old Ross broiler chicks were obtained from commercial poultry company. Chicks were raised in separated rooms and were provided with water and feed *ad libitum;* all chicks were vaccinated against avian influenza H5N1 and infectious bursal disease virus at 10 days old and no vaccination for NDV was used. Chicks were divided into four groups (25 per each) as follow; control non infected group (group 1), infected group at 10 days old (group 2), infected group at 20 days old (group 3) and infected group at 30 days (group 4). The infected groups were inoculated intraocular with a dose 10^6 EID_{50} of NDV (in a total volume of 0.1 ml). Birds were monitored every day for abnormal clinical signs. Three birds from each group were sacrificed at 1, 3, 5- and 7-days post inoculation (dpi). Serum and nervous tissue samples were collected from all experimental groups. This experimental protocol was approved by Institutional Animal Care and Use Committee (IACUC), Cairo University, Egypt (Approval number, CU/II/F/65/17).

Evaluation of serum virus-specific antibodies (Haemagglutination inhibition (HI))

Serum samples were collected for HI test, which was carried out according to OIE, (2012) using 1% freshly prepared chicken RBCs suspension. Serum samples were collected from control group divided to control a (10 days old) for comparison with group 2 (10 days), control b (20 days old) for comparison with group 3 (20 days old) and control c (30 days old) for comparison with group 4 (30 days old).

Histopathology and immunohistochemistry

Nervous tissue samples included the brain and spinal cord which was divided into three segments; cervical, thoracic and lumbar segments and sciatic nerves, preserved in neutral buffered formalin 10% and routinely processed, sectioned and stained with Hematoxylin and Eosin (H&E) (Bancroft 2013). Tissue sections were examined using Olympus BX43 light microscope and

captured using Olympus DP27 camera linked to Cellsens dimensions software (Olympus).

For immunohistochemistry, Paraffin blocks were collected at the 7th dpi from different groups. Hyperimmune serum against NDV was raised in rabbit using series of injections following the schedule (Samiullah *et al.* 2006). Antibody purification was performed using Magne™ Protein G Beads for Antibody Purification according to the manufacturer's instructions. Tissue sections were obtained on Poly-L-Lysine coated slides, then they were deparaffinized and rehydrated, antigen retrieval was performed by heat induction, blocking of non-specific protein binding and endogenous peroxide was followed by overnight incubation in primary antibody (Rabbit anti NDV Ig) then incubated with horseradish peroxidase–conjugated goat polyclonal secondary antibody to rabbit Ig (SM802 EnVision™ FLEX /HRP). The color was developed with 3, 32 - Diaminobenzidine (DAB) substrate (DM827 EnVision™ FLEX DAB+ Chromogen) and counterstained with Mayer's hematoxylin. Exclusion of primary antibodies was used for negative control (Burns *et al.* 2005).

The selected brain and spinal cord sections from group 4 (30 days old) at 7 dpi were stained by luxol fast blue (American Mastertech, CA, USA) according to Bancroft (2013).

Statistical analysis

Statistical analyses were performed using one-way factorial analysis of variance (ANOVA). Statistical significance was defined as ($p \leq 0.05$) using SPSS 17.

RESULTS AND DISCUSSION

Serum NDV specific antibody titers

Results of antibody titers against NDV were summarized in Table (1). At 1 dpi, there was a significant decrease in antibody level in group 4 (0.00 ± 0.00) compared with group 2 (3.66±0.88). At 3 dpi, there was a significant increase in antibody level in group 2 (3.66± 0.33) compared with group 3 and group 4 which scored (1.00± 0.00) and (0.66 ± 0.33) respectively. No significant difference was detected among all infected groups at 5 dpi. While at 7 Dpi group 3 exhibited the highest immunological response and reached the protective antibody titer level (8.66± 1.33) (Fig. 1).

Clinical nervous signs and mortality rate

The nervous signs were restricted to 20-and 30-days old groups (groups 3 and 4). General clinical signs began at 5 dpi in the form of severe depression and ruffled feathers which increased in severity at 7 dpi. The most obvious nervous signs included muscular tremors, crouched head, paresis and paralysis (Fig. 2a to 2d). No mortalities were detected at 10 days old infected birds (group 2), while mortalities were 8% at 20 days old group and reached to a maximum of 24% at 30 days old group. The non-infected control chickens appeared normal along the experimental period.

Histopathological findings

The scoring of different neuro-histopathological lesions in different ages, duration of infection and areas of nervous was illustrated in Table 2.

Concerning the cerebrum, the lesions in 10 days old age group beginning at 1dpi and were more in cerebral grey matter than white matter, which included capillary congestion, mild meningeal hemorrhages and scarce glial cell and endothelial capillary proliferation (Fig. 3a). The lesions developed at 3 and 5 dpi to include perivascular lymphocytic cuffing and vasculitis of individual blood

Table 1. NDV mean antibody titers with respect to days post infection by HI test expressed as Log 2.

Groups	1 dpi	3 dpi	5 dpi	7 dpi
Control a	5.33 ± 0.88 [c]	4.66 ± 0.66 [c]	3.00± 0.57[b]	4.00 ± 1.00[c]
Group 2	3.66±0.88 [b, c]	3.66±0.33[c]	2.66± 0.66[b]	3.33 ± 0.33 [b, c]
Control b	1.66 ± 0.88 [a, b]	2.33± 0.33[b]	1.66± 0.33 [a, b]	1.00 ± 0.00 [a, b]
Group 3	1.66 ± 0.33 [a, b]	1.00± 0.00 [a]	2.33 ± 0.66 [a, b]	8.66± 1.33[d]
Control c	1.00 ± 0.00[a]	0.66 ± 0.33[a]	0.33 ± 0.33[a]	0.00 ± 0.00[a]
Group 4	0.00 ± 0.00[a]	0.66 ± 0.33[a]	2.00 ± 1.00[a, b]	2.33 ± 1.20[a, b, c]

*Values are expressed as mean of Log 2 ±SE.

a, b, c and d indicate significant different between values within the same column.

Control a= non infected group of chickens of 10 days age.

Control b= non infected group of chickens of 20 days age.

Control c= non infected group of chickens of 30 days age.

Table 2. Qualitative scoring of neuropathological lesions in cerebrum, cerebellum and spinal cord.

Age of broiler chicken (days)	10				20				30			
Days post infection (dpi)	**1**	**3**	**5**	**7**	**1**	**3**	**5**	**7**	**1**	**3**	**5**	**7**
Cerebrum												
Gliosis	+	+	++	+++	+	+	+++	+++	+	++	+++	+++
Endothelial capillary proliferation	-	+	+	++	-	+	++	++	+	+	+	+
Perivacular cuffing	-	+	++	++	-	+	++	+++	-	-	+	++
Cortical malacia	-	-	-	++	-	-	-	-	-	-	-	-
Striatum and medullary malacia	-	-	-	-	-	-	-	++	-	-	++	+++
Cerebellum												
Gliosis	+	+	+	+++	+	+	++	++	+	+	+	++
Purkinje cells necrosis	-	-	-	+	-	-	+	++	-	-	+	+++
Malacia	-	-	-	-	-	-	-	+	-	-	+	++
Demyelination of cerebellar nuclei	-	-	-	-	-	-	+	++	-	-	++	+++
Perivascular cuffing	-	-	-	+	-	+	++	++	-	-	++	+++
Spinal cord **Cervical**												
Chromatolysis	-	-	+	+	-	-	+	++	-	-	++	+++
Demyelinationand axonal spheroids formation	-	-	-	-	-	-	+	++	-	-	++	+++
Perivascular lymphocytic cuffing	-	-	-	-	-	-	+	++	-	-	++	++
Thoracic												
Chromatolysis	-	-	-	+	-	-	-	+	-	-	+	+
Demyelination and axonal spheroids formation	-	-	-	-	-	-	-	+	-	-	+	++
Perivascular lymphocytic cuffing	-	-	-	-	-	-	+	+	-	-	+	++
Lumbar												
Chromatolysis	-	-	-	++	-	-	+	+++	-	-	++	+++
Demyelination and axonal spheroids formation	-	-	-	-	+	-	-	++	-	-	++	+++
Perivascular lymphocytic cuffing	-	-	-	++	-	-	++	+++	-	-	+++	+++

Mild focal (+), moderate (++), severe diffuse (+++).

vessels in the cerebral cortex (Fig. 3b) while typical picture of non-suppurative encephalitis comprising the cerebral cortex became evident at 7 dpi. Microscopically, the lesions were characterized by lymphocytic infiltration in meningeal and perivascular areas, neuronal degeneration, microgliosis, endothelial capillary proliferation, vasculitis (Fig. 3c and 3d) and development of foci of encephalomalacia in cerebral grey matter in sub-leptomeningeal area (Fig. 3e). The reaction extended deeper into hypothalamus. Meanwhile, no lesions involving the medulla or mid brain could be detected in this age group. Neuropathological lesions at 20 days old birds showed variation in lesions severity among the different durations of infection. Mild lymphocytic meningitis was early detected at 1 dpi in this group, mild gliosis and endothelial capillary proliferation were evident in cerebral cortex. The lesions developed in 3 dpi to perivascular cuffing in cerebral cortex while the

neuropathological lesions developed at 5 dpi to give the previously described picture of non- suppurative encephalitis but with moderate severity (Fig. 3f) and become more diffuse in cerebral cortex. Severe diffuse meningoencephalitis was detected at 7 dpi of this age group. The cerebral cortex showed perivascular cuffing involving different blood vessels with diffuse gliosis and neuronal degeneration (Fig. 3g) involving different areas of cerebral cortex. The brain stem showed gliosis, spongiosis with demyelination and neuronal necrosis (Fig. 3h). Moreover, inflammation of the choroid plexus with vacuolization and necrosis of ependymal cells were also noticed (Fig. 3i). Concerning neuropathological lesions developed in infected chickens at 30 days old, lesions were more or less similar to lesions described at 20 days old group at 1 and 3 dpi. While at 5 dpi, mild gliosis was evident in cerebral grey matter and more severe in striatum (Fig. 3j). Lesions at 7 dpi involved the cerebral cortex and medulla. The lesions developed in cerebral cortex were similar to 20 age group at 7 dpi but with less severe perivascular cuffing, status spongiosis and diffuse gliosis. The reaction became more chronic and involving the striatum and the medulla oblongata. The striatum showed hyalinization of vascular walls (Fig. 3k) while the medulla oblongata showed microgliosis, demyelination and neuronal degeneration (Fig. 3l).

Lesions in cerebellum at 10 days old group were mild at 1, 3 and 5 dpi and included mild gliosis of molecular layer and congestion involving the cerebellar medulla. While moderate gliosis of the molecular layer, small perivascular lymphocytic aggregation in pericapillary areas were detected with endothelial hypertrophy (Fig. 4a) at 7 dpi. Lesions at 20 days old at1dpi were mild gliosis and endothelial capillary proliferation that were evident in molecular layer of cerebellum (Fig. 4b). The lesions developed at 3 and 5 dpi to perivascular cuffing in cerebellar medullary area (Fig. 4c). Moreover, the histological changes were more severe at 7 dpi and included focal malacia of molecular layer with diffuse gliosis and necrosis of individual purkinje cells (Fig. 4d), in addition the cerebellar nuclei showed neuronal degeneration, microgliosis, axonal spheroids with demyelination and small perivascular lymphocytic cuffing (Fig. 4e).The cerebellar lesions were mild (1 dpi) to moderate (3 and 5 dpi) in chickens from group 4 (30 days age group), including endothelial capillary proliferation, gliosis, and perivascular cuffing in cerebellar folia. Severe histopathological lesions were detected at 7 dpi, multiple areas of encephalomalcia of molecular layer as well as cerebellar folia, necrosis of purkinje cells associated with gliosis were recorded in examined sections (Fig. 4f). There were areas of spongiosis and demyelination in the cerebellar molecular layer with microgliosis (Fig. 4g). Central chromatolysis and marked necrosis of the ganglionic cells of the cerebellar nuclei associated with gliosis, demyelination, axonal spheroids formation, and perivascular lymphocytic cuffing were evident (Fig. 4h and 4i).

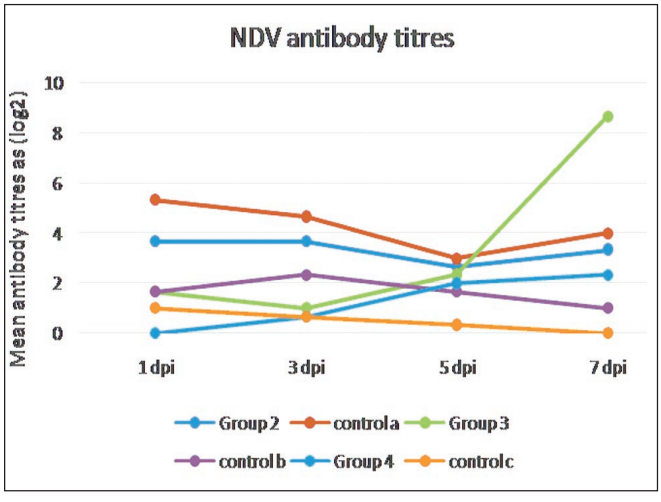

Fig. 1. NDV mean antibody titers with respect to days post infection by HI test.

[Each point represents the mean HI titers (log 2) of serum (n = 3); Control a = non infected group of chicken of 10 days age. Control b = non infected group of chickens of 20 days age. Control c = non infected group of chickens of 30 days age.]

The neuropathological lesions in the different segments of spinal cord (cervical, thoracic and lumbar segments) varied according to the age of birds and duration of infection. The lesions at 10 days age group after 1 and 3 dpi were mild and including congestion of blood capillaries comprising the grey matter of lumbar spinal cord. Additionally, perivascular few lymphocytic aggregations in the meningeal area of the lumbar region and mild gliosis of spinal grey matter were detected at 5 dpi. Lesions at 7 dpi give the picture of non-suppurative myelitis that was noticed in thoracic and lumbar spinal cord, the lesions described as mild perivascular lymphocytic cuffing in the grey and white matter associated with massive gliosis and lymphocytic infiltration (Fig. 5a). On the other hand, lesions in birds at 20 days old group (1 and 3 dpi) included mild gliosis and endothelial capillary proliferation involving the different segments of spinal cord as well as satellitosis of individual neurons in the grey matter. At 5 dpi, the cervical spinal cord showed astrocytosis of white matter and gliosis with endothelial capillary proliferation of grey matter of thoracic and lumbar spinal segments (Fig. 5b). Severe picture of non-suppurative myelitis was detected

Fig. 2. Chickens experimentally infected with NDV (20 days old).
[Showing crouching, dropped wings (a) and paresis (b) at 5 dpi, severe depression and paralysis (c), reluctance to move with dropped neck and head as well as paralysis at 7 dpi (d)].

at 7 dpi. Moreover, marked inflammatory reaction was observed in meninges of cervical spinal cord (Fig. 5c) and extended into cervical spinal white matter (Fig. 4d) with axonal spheroids (Fig. 5e), chromatolysis, neuronal necrosis, marked gliosis and perivascular lymphocytic cuffing were detected in grey matter. The inflammatory reaction and marked neuronal necrosis were more severe in the lumbar spinal cord associated with severe perivascular cuffing (Fig. 5f and 5g) and demyelination. Lesions of moderate severity were detected in the thoracic spinal cord. Concerning neuropathological lesions developed in infected chickens at 30 days old lesions were more similar to 20 days old group at 1 and 3 dpi, while early demyelination of cervical spinal segments with the appearance of axonal spheroids and gliosis were detected (Fig. 5h). Moderate perivascular lymphocytic cuffing involving both gray and white matter was observed in the cervical segments (Fig. 5i), the lesions were less severe in thoracic and lumbar spinal cord segments. At 7 dpi, severe lesions were evident in lumbar spinal cord and characterized by marked neuronal necrosis and astrogliosis (Fig. 5j). Marked demyelination, axonal spheroids formation (Fig. 5k) and perivascular lymphocytic cuffing of grey and white matter (Fig. 5l) were observed in examined sections, the lesions were less in cervical and thoracic spinal segments.

Incidence of demyelination in different areas of central nervous system (Luxol fast blue)

The demyelination was prominent in striatum, (Fig. 6a), cerebellar molecular layer (Fig. 6b), cerebellar nuclei, white and grey matter of spinal cord at 30 days age group at 7 dpi (Fig. 6c and 6d).

Immunohistochemical findings

The immunohistochemical characterization of NDV viral antigen in different areas of nervous system of 30-day old chickens at 7 dpi revealed weak expression of the virus antigen in nerve cells of the cerebrum and became more in the glia cells. In addition, virus antigen was detected in the wall of blood capillaries of the choroid plexus and the infiltrating lymphocytes (Fig. 7a) and Purkinje cells of cerebellum. The associated endothelial lining blood capillaries and glia cells were the sites for

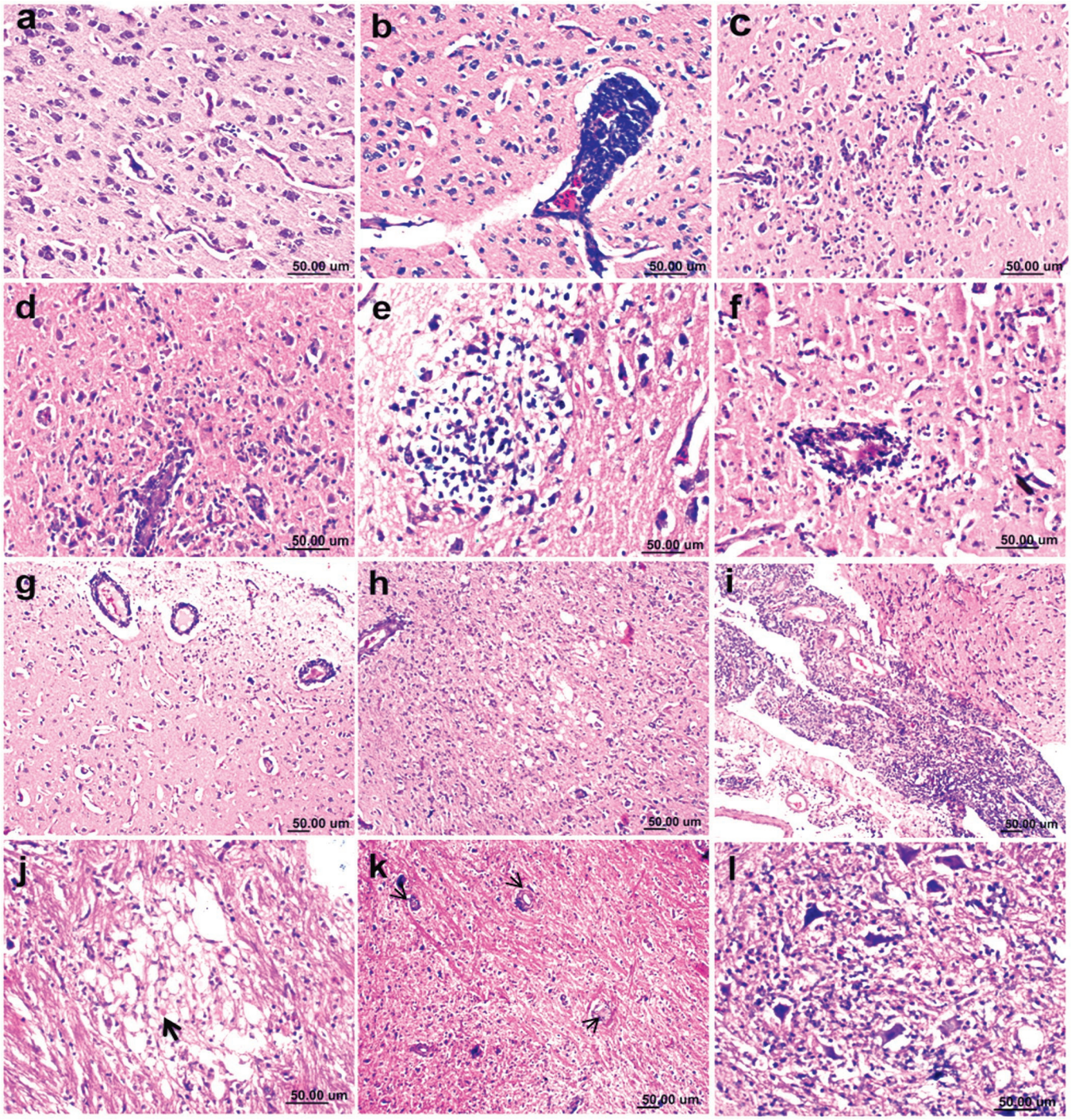

Fig. 3. Histological H&E stained sections from different cerebrum areas in NDV experimentally infected chicken.
[a) Mild endothelial capillary proliferation and gliosis of hyperpallium (10 days old,1 dpi). b) Vasculitis and perivascular lymphocytic cuffing of mesopallium (10 days old, 3 dpi). c) Focal microgliosis, neuronal vacuolization and necrosis of nidopallium associated with satellitosis (10 days old,7 dpi). d) Marked endothelial capillary proliferation, massive neuronal necrosis with karyorrhectic debris with diffuse gliosis of nidopallium (10 days old,7 dpi). e) Encephalomalacia note the spongiosis and microgliosis in the sub-leptomeningeal area (10 days old,7 dpi). f) Vasculitis and thrombosis with diffuse gliosis in mesopallium (10 days old, 5 dpi). g) Perivascular lymphocytic cuffing associated with thrombosis, endothelial capillary proliferation and perivascular edema of microvasculature and gliosis in the pallidum (20 days old, 7 dpi). h) Spongiosis, demyelination and gliosis with hyalinized vascular wall of brain stem (20 days old,7 dpi). i) Diffuse lymphocytic infiltration in the choroid plexus with vacuolization and necrosis of ependymal cells (20 days old, 7 dpi). j) Severe demyelination and spongiosis of striatum with axonal spheroid formation (arrow) (30 days old, 5 dpi). k) Hyalinization of vascular wall (arrow) with gliosis of striatum (30 days old, 7 dpi). l) Neuronal degeneration, microgliosis and demyelination of medulla oblongata (30 days old, 7 dpi)].

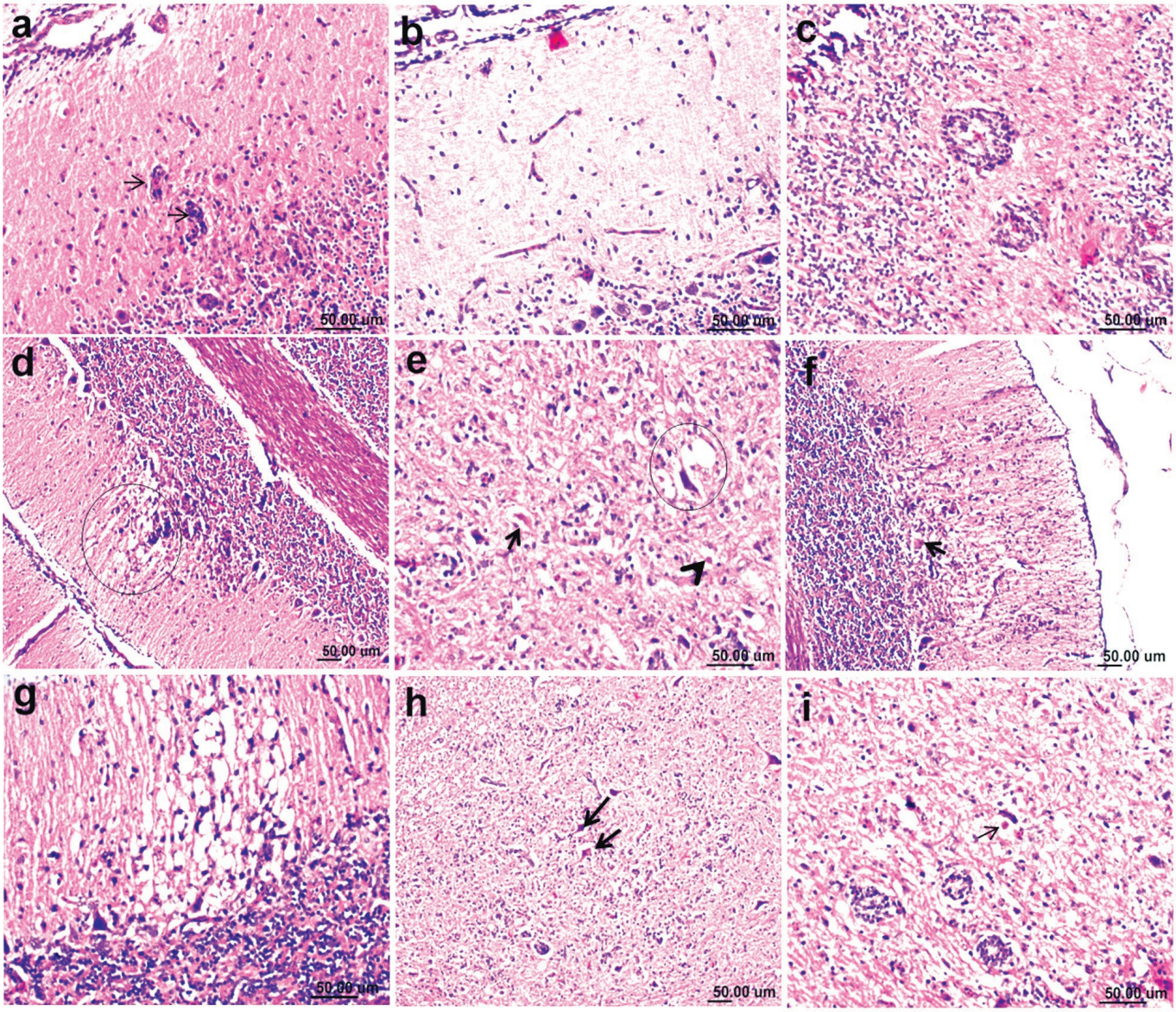

Fig. 4. Histological H&E stained sections from different cerebellum areas in NDV experimentally infected chickens.
[a) Diffuse gliosis, perivascular lymphocytic aggregation with endothelial proliferation of vascular capillary wall (arrow) in the molecular layer (10 days old ,7 dpi). b) Mild gliosis of molecular layer with endothelial capillary proliferation (20 days old,1 dpi). c) Perivascular lymphocytic cuffing of the medullary core of cerebellar folia (20 days old, 5 dpi). d) Focal encephalomalcia (circle), gliosis and individual purkinje cell necrosis (20 days old,7 dpi). e) Demyelination (circle) with axonal spheroid formation (arrow head) and neuronal necrosis(arrow) with diffuse gliosis of cerebellar nucleus (20 days old, 7 dpi). f) Necrosis of purkinje cells (arrow) with diffuse gliosis of molecular layer (30 days old,7 dpi). g) Demyelination and microgliosis of molecular layer associated with loss of purkinje cells (30 days old, 7 dpi). h) Spongiosis, demyelination, necrosis of motor neurons (arrow) and diffuse gliosis of cerebellar nucleus (30 days old, 7 dpi). i) Severe demyelination with axonal spheroid formation (arrow) with perivascular lymphocytic cuffing of cerebellar nucleus (30 days old, 7 dpi)].

virus antigen deposition of moderate expression (Fig. 6b and 6c). Intense virus antigen deposition was evident in spinal cord in neurons, glia cells, vascular endothelium and in the lymphocytic cuffs (Fig.7d).

The present work gives an overview on the distribution and progression of neuropathological lesions in the nervous system of chickens experimentally infected with vNDV via intraocular instillation at different ages. The nervous signs were mild at 10-day old chicks and the severity increased at 20 and 30-day old ages appeared earlier in older age group. these findings were supported by the virus titer and histopathological findings that were directly proportional in severity with age, it was assumed that restriction of nervous signs in older age to the

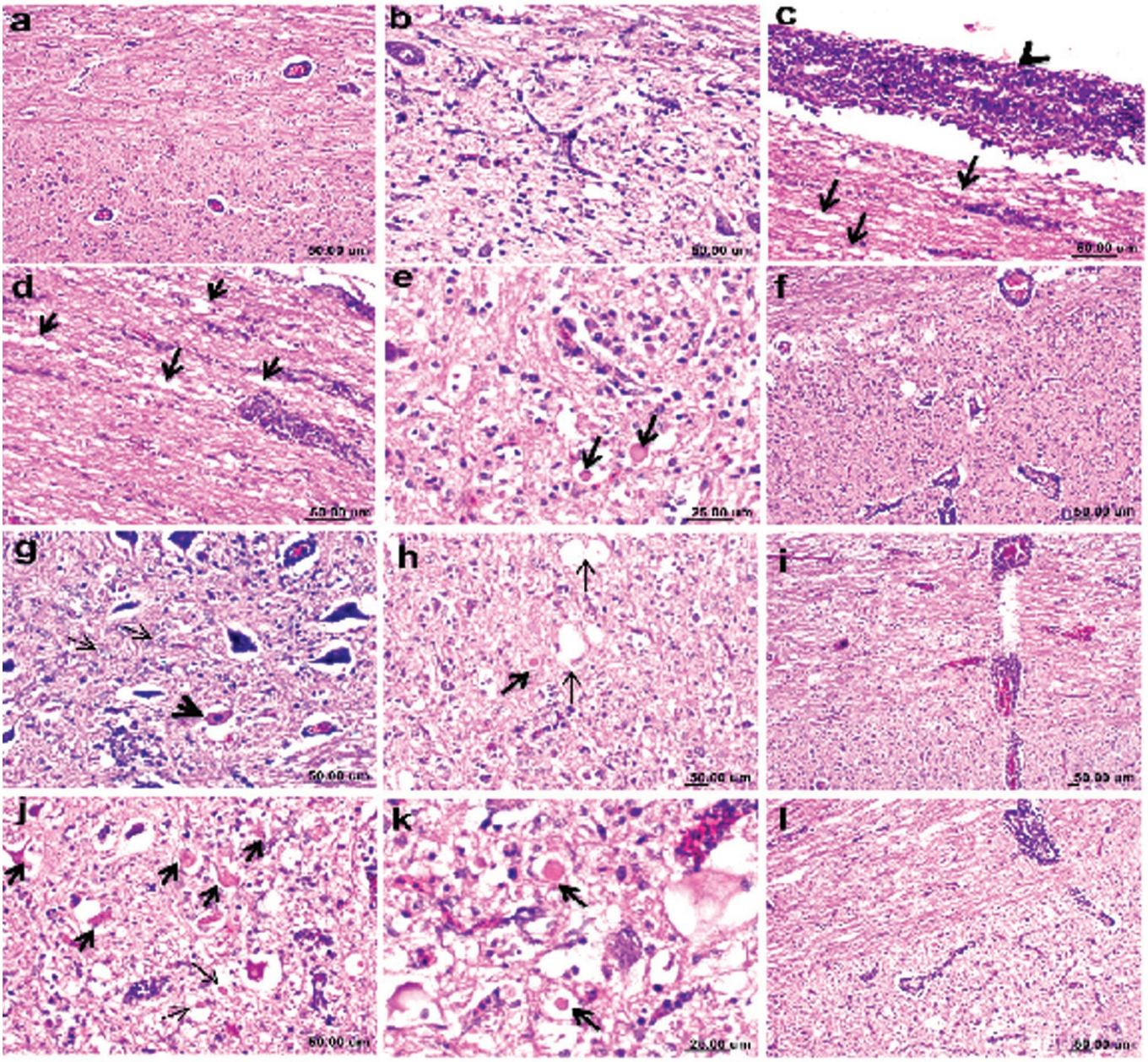

Fig. 5. Histological H&E stained sections from different spinal cord segments in NDV experimentally infected chickens. [a) Mild perivascular lymphocytic cuffing in one row in white and grey matter of the lumbar spinal segment (10 days old, 7 dpi). b) Focal demyelination and diffuse gliosis with mononuclear cell infiltration of grey matter of cervical spinal segment (20 days old, 5 dpi).c) Meningeal infiltration of lymphoplasmacytic cells (arrow head) of cervical segment with demyelination of white matter (arrow) (20 days old, 7 dpi). d) Lymphoplasmacytic infiltration in the white matter of cervical spinal cord that showing demyelination (arrow) (20 days old,7 dpi). e) Demyelination with axonal swellings (spheroids) (arrow) associated with microgliosis of grey matter of cervical spinal cord (20 days old,7 dpi). f) Vasculitis with perivascular lymphocytic cuffing and diffuse gliosis of both grey and white matter of lumbar segment (20 days old,7 dpi). g) Necrosis and karyorrhectic nuclear changes with complete chromatolysis of motor neurons (arrow head), hypertrophied astrocytes (arrow) and astrogliosis in lumbar segment (20 days old, 7 dpi). h) Demyelination (thin arrow) and axonal spheroids (thick arrow) of cervical grey matter (30 days old, 5 dpi). i) Moderate lymphoplasmacytic vascular cuffing comprising the white and grey matter of cervical segment (30 days old, 5 dpi). j) Diffuse severe necrosis and of motor neurons (thick arrow) associated with astrogliosis, severe demyelination and axonal spheroid formation (thin arrow) of lumbar grey matter (30 days old, 7 dpi). k) Multiple axonal spheroids(arrow) with severe demyelination of lumbar grey matter (30 days old, 7 dpi). l) Intense vasculitis and perivascular cuffing of both white and grey matter of lumbar segment (30 days old, 7 dpi)].

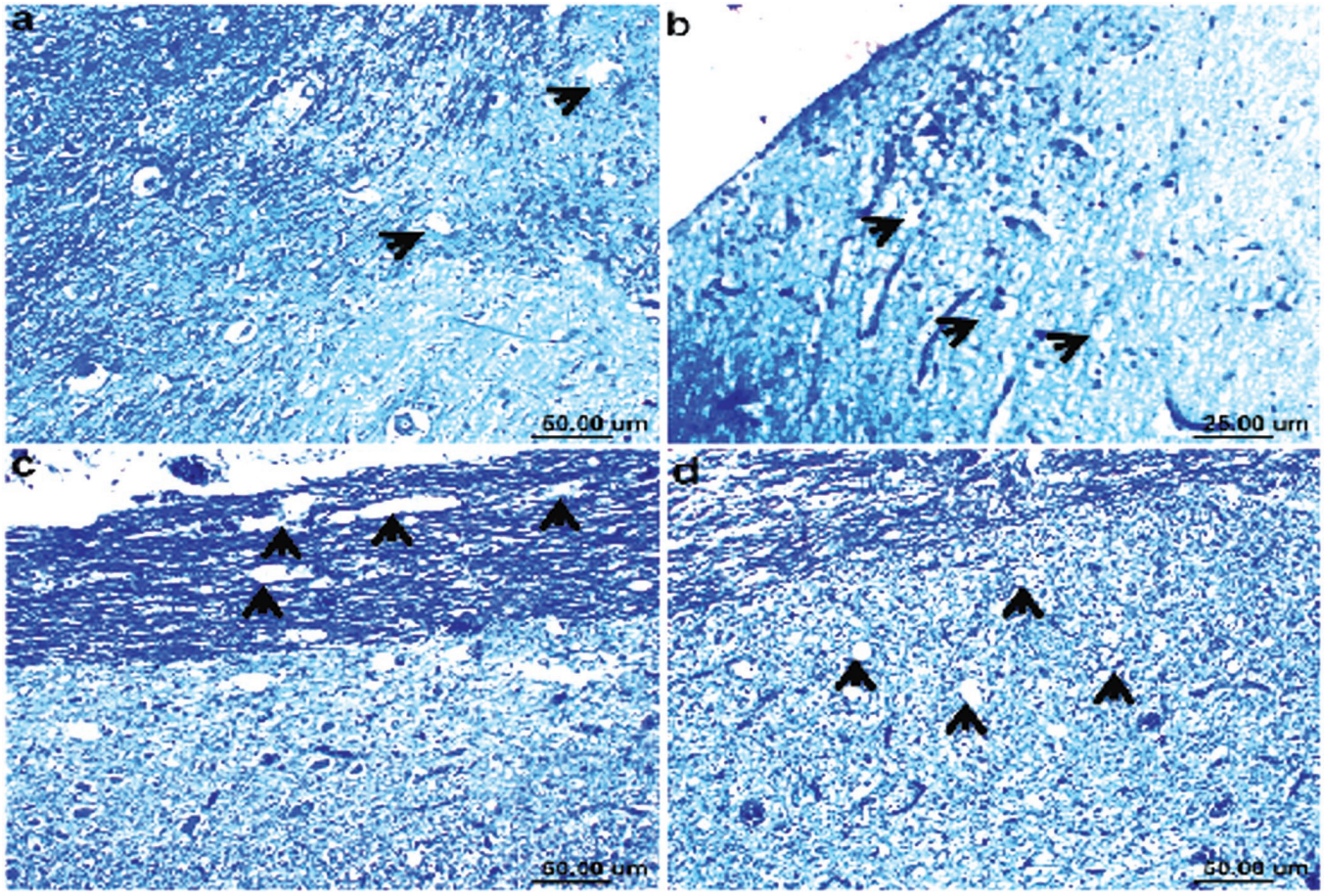

Fig. 6. Histological Luxol fast blue stained sections from chicken experimentally infected with NDV of 30 days old.
[At 7 dpi, showing demyelination (arrows) comprising striatum (a), the molecular layer of cerebellum (b), white matter (c) and grey matter of lumbar spinal cord (d)].

reduction in immune status with advancing age versus the presence of maternal immunity in young age chicks as confirmed by Anis *et al.* (2013) and Mousa *et al.* (2019), in addition the appearance of clinical signs begun at 5 to 7 dpi of 20 and 30 age groups indicate the slower onset of virus progression in the C.N.S. Brown *et al.* (1999) found that birds infected by neurotropic vNDV remained alert then become depressed and developed paresis or paralysis at 5 days or later of infection. The intraocular instillation of NDV induced neuropathological lesions in the CNS, Cattoli *et al.* (2011) are viewed that experimental aerosolization of birds by high NDV doses induced lesions in upper respiratory tract preferentially than the nervous system. However, there was variation in severity and distribution of microscopic picture of vNDV induced neuropathology in relation to the bird age. In young age group (10 days old), the cerebral cortex was the most affected area in this group compared with medulla, cerebellum and spinal cord and the typical picture of encephalitis was detected at 7 dpi with development of vasculitis and focal area of encephalomalacia with lesion in cerebellum was restricted to molecular layer not extended into the cerebellar nucleus. Moreover, gliosis and endothelial capillary proliferation with perivascular cuff with no areas of cerebellar malacia were evident. While only the thoracic and lumbar segments showed perivascular lymphocytic cuffing with no additional changes in spinal cord indicating neurodegeneration or demyelination. These lesions reflected the lack of paresis or paralysis in birds of this age group while the neuroinflammation of cerebrum did not affect the activity of birds either in feeding or behavior. On the contrary, the difference in behavior and consciousness of birds between 20 and 30 age groups was related to the degree of status spongiosis and demyelination of cerebrum, striatum and medulla oblongata, as these changes were severe at 30 age group compared with 20 age group resulted in severe depression, recumbency and off food in birds of 30 days age, in contrast to alertness of birds with normal feeding behavior

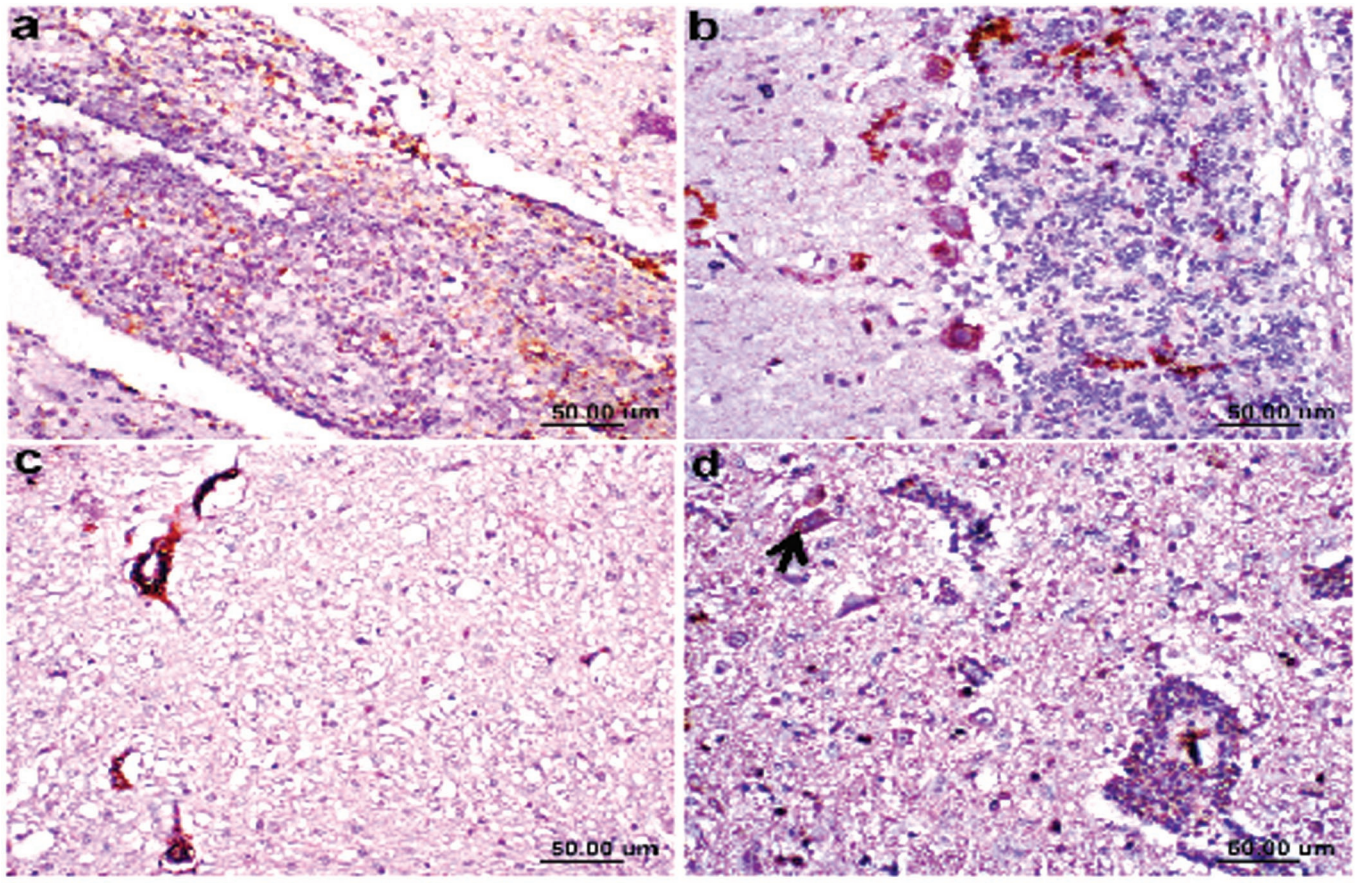

Fig.7. Immunohistochemical stained sections of chicken experimentally infected with NDV.
[At 30 days old and 7dpi, showing NDV viral antigen expression in the lymphocytes and ependymal cells of choroid plexus (a), in purkinje cells and vascular endothelial capillary of cerebellum (b), vascular endothelium and glia cells in the cerebellar nucleus (c), in motor neuronal cytoplasm (arrow), lymphocytes and in microglia cells (d)].

in spite of paresis and paralysis that developed in birds at 20 days old age group.

The cerebellum and spinal cord were the most areas of CNS developed severe lesions of inflammatory and degenerative nature with formation of axonal spheroids and associated with marked demyelination. The malacia, status spongiosis, and demyelination were detected at 7 dpi of 20 days old birds but these lesions developed earlier at 30 days age group (5 dpi) and reflected the appearance of torticollis, dropped head, tremors, paresis and paralysis in birds of older ages. Moreover, the result showed that paresis and paralysis induced by NDV infection is related to spinal cord involvement that was more severe in cervical and lumbar segments not due to sciatic nerve lesions as no lesions were detected in the sciatic nerves in all ages groups, on contrast, Nakamura *et al.* (2008) found virus antigen in peripheral sciatic nerves. No available literatures discussed the difference of NDV dissemination in different segments of spinal cords. Ecco *et al.* (2011) stated that infection of birds with VNDV induced nervous signs and lesions that begun at 14 days of age and reach peak at 21 days and developed at 5 dpi and severe lesions was detected at 10 dpi.

Perivascular cuffing was detected in all age groups that composed of lymphocytes in 10 days age group and was restricted to cerebral cortex and hypothalamus, small capillaries of cerebellar molecular layer and lumbar spinal cord with no additional reaction could be seen in mild brain, medulla and cerebellar nucleus. This reflects the presence of maternal immunity that neutralize virus and limits its replication resulting in mild and focal distribution of neuropathology, this was confirmed by the antibody serum titer. Moura *et al.* (2016) attributed the mild perivascular lymphocytic inflammation in day-old chickens to immature immune system that did not allow severe lymphocytic inflammation compared with mature birds.

The perivascular cuffing with lymphocytes admixed with plasma cells was detected in that spinal cord segments that reflects the developed immune response

at this age which tried to reduce virus replication and spread in the CNS, this was supported by our antibody titer which was higher at this age group. While at 30 days old group, the perivascular cuffing with lymphocytes was scarce in cerebrum moderate in cerebellum and lumbar spinal cord, this reflects the immune suppression status of the birds at this age with widespread virus replication and diffuse neuropathological lesions in different areas of CNS and lower antibody titer that did not provide protection against NDV dissemination. This result was previously recorded by Lu *et al.* (2014) who stated that lower antibody titer against NDV induced lack of protection against NDV infection. The presence of marked neuronal necrosis in cerebral cortex, status spongiosis of striatum and demyelination of medulla oblongata, molecular layer of cerebellum, cerebellar nucleus and spinal cords indicate complete tract degeneration that was hallmark of neuropathology of NDV of old ages days age group at 7 dpi) as confirmed by Bhaiyat *et al.* (1994), in addition they proved the hematogenous ND viral dissemination into CNS that was detected in the present work and was confirmed by perivascular viral positive stained lymphocytes and localization of the virus antigen in vascular endothelium by IHC. Moreover, the presence of inflammatory reaction in choroid plexus and periventricular area proves the dissemination of NDV via cerebrospinal fluid. We assumed that NDV induced demyelination in CNS of infected bird of secondary type, the possible pathways of demyelination are two, firstly, neuronal necrosis and axonal degeneration due to direct viral infection and selective replication in motor neurons of cerebellum and spinal cord, that proved by virus antigen localization in neurons by IHC with subsequent secondary demyelination to axonal injury as reviewed by Sarma (2010). The author stated that axonal damage might develop secondary to myelin damage (outside-in model) or myelin damage developed secondary to axonal injury (inside–out model). Secondary, NDV induced myelitis resulting in secondary demyelination as we detect severe demyelination in spinal cord in association with severe lymphocytic vascular cuffing and massive gliosis. Kornek *et al.* (2000) stated that infection and neurotropic strains of Mouse hepatitis virus (MHV) induce demyelination that begins as early as day 5 post inoculation which was concurrent with acute inflammation, in addition Wu *et al.* (2000) found that macrophages and T cells modulate pathologic changes of demyelination induced by MHV.

The malacia developed in the present work differed in severity and distribution in different age group, the lesion was small, focal and restricted to cerebrum of 10 days old birds which assumed to be related to vascular lesions that results in ischemia, while diffuse severe malacia that was detected in cerebellum, medulla was related to in part to vascular hyalinization and stenosis that were evident at older age as well virus replication in astrocytes, purkinje cells and neurons of spinal cord that confirmed by virus antigen residence in immunohistochemistry. Bhaiyat *et al.* (1994) found that young chicks that were experimentally infected by VNDV developed malacia in brain that was related to severe inflammation of cerebrum, while occlusive vasculitis and vascular fibrosis with subsequent brain ischemia were detected in late stage of encephalitis in NDV infection was previously described by Jubb and Huxtable (1993).

In our results, gliosis was detected in all age groups but reactive astrogliosis become more prominent in older age groups and was detected in areas of malacia and demyelination in the cerebellum and spinal cord. We assumed the astrogliosis developed in NDV was related to a reparative process secondary to degenerative and necrotic process in neuronal parenchyma (Maxie 2007) in addition to virus replication in astrocytes as confirmed by NDV antigen residence in astrocytes by IHC. Ecco *et al*. (2011) considered the direct virus infection of astrocytes as the prime stimuli for astrogliosis in chickens infected by velogenic NDV.

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